THE ISOLATION OF A LOW MOLECULAR WEIGHT PROTEIN INVOLVED IN THE ENERGY TRANSDUCTION FROM COMPLEX I TO THE ATP SYNTHASE

R. KIEHL[+] and E. BÄUERLEIN*

Max-Planck-Institut für medizinische Forschung, Abteilung Naturstoff-Chemie, D-6900 Heidelberg, FRG

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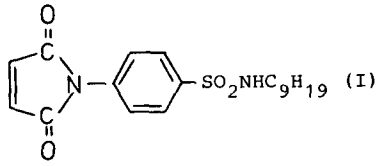
1. Introduction

We reported recently that in beef heart mitochondria the lipophilic maleimide NSPM (I) inhibited State 4 → State 3 transition, if glutamate + malate



(I)

or β-hydroxybutyrate were the substrates, whereas with succinate State 4 was stimulated to 90% of State 3 respiration [1]. Concomitantly with the inhibition uncoupling by DNP or CCCP was prevented. For the incubation of well-coupled mitochondria with 6.9 nmol NSPM/mg protein for 5 min caused complete inhibition, the same concentration was used for the labelling experiments with [^{14}C]NSPM. After the extraction with ethanol, removal of the lipids and purification on Sephadex LH-20 a water soluble, [^{14}C]NSPM-binding protein of low mol. wt (< 13 000) was isolated which is very probably responsible for the specific inhibitors of Site I coupling.

Abbreviations: NSPM, *N'*-(*N''*-*n*-nonyl-4-sulfamoylphenyl)-maleimide; ASPM, *N'*-(*N''*-acetyl-4-sulfamoylphenyl)-maleimide; DNP, 2,4-dinitrophenol; CCCP, carbonylcyanide 3-chlorophenylhydrazone; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; t.l.c., thin-layer chromatography

[+] New address: Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037, USA

*To whom correspondence should be addressed

2. Materials and methods

2.1. *Isolation of mitochondria*

Intact beef heart mitochondria were isolated according to the method of Smith [2], modified by the use of subtilisin [3], to obtain high respiration control ratios. In a second method the mitochondria were prepared by the use of a high-speed blender according to Blair [4], to avoid subtilisin; in this preparation light and heavy mitochondria were separated. The protein concentration was determined by the biuret method in the presence of 0.33% desoxycholate [5].

2.2. *Measurement of respiration*

Oxygen uptake was measured with a Clark-type oxygen electrode. All mitochondrial preparations were checked for structural integrity using the criterion of respiratory control [6].

2.3. *Chemicals and reagents*

N'-(*N''*-*n*-nonyl-4-sulfamoylphenyl)-maleimide, NSPM (I), was synthesized [7] according to the ASPM preparation of Merz et al. [8]. A microsynthesis of [^{14}C]NSPM (spec. act. 3.73 mCi/nmol) was developed [7]; the pure maleimide (m.p. 92°C; ultraviolet max 244 nm) was obtained without any further preparation in quantitative yield from its open form, *N'*-[1,4-^{14}C]maleoyl-*N''*-*n*-nonylsulfanilamide (m.p. 168°C; ultraviolet max 285 nm). The product is stable for 1 h in ethanol or dimethylformamide. The sources of the reagents were: Buchler Amersham, [1,4-^{14}C]-maleic anhydride; Novo Industries, Mainz, subtilisin Novo; Sigma, München, CCCP; Protosol, [^{14}C]toluene, NEN.

2.4. *Rapid polyacrylamide gel electrophoresis at pH 5.0*

PAGE for mitochondrial proteins, as described by Cattel et al. [9], was varied by using 1% SDS in 0.1 M sodium acetate buffer pH 5.0 for the preparation of 10% gels; samples of membranes were dissolved in a solution containing (final concentration) 3% (w/v) SDS, 5 mM EDTA and 0.1 mM sodium acetate buffer pH 5.0, but without DTT, to avoid reaction products of [^{14}C]NSPM with DTT as well as the formation of the related miscelles in PAGE. The electrophoresis was carried out at 6 mA/gel in a reservoir buffer of 0.1 M sodium acetate pH 5.0 containing 1% (w/v) SDS and was completed within 3 h.

2.5. *Incubation of beef heart mitochondria with [^{14}C]NSPM and isolation of the NSPM-binding protein*

As example for six preparations of mitochondria – three with the subtilisin and three with the high-speed blender method – one of the first group is described (scheme 1): The mitochondria were distributed on four centrifuge tubes (Beckman rotor Ti 60). [^{14}C]-NSPM was dissolved in 1 ml ethanol and the aliquots were added immediately to the four portions of mitochondria under subsequent vigorous mixing by a whirl mix (Cenco) for 5 min at 20°C. After standing over night in the refrigerator, each portion was extracted three times with 25 ml ethanol for 15 min at 20°C on the whirl mix and centrifuged for 5 min at 8000 × *g* (Beckman rotor Ti 60, 10 000 rev/min). The 300 ml supernatant was filtered and evaporated to dryness at 30°C i.vac. Samples of the mitochondria before and after the extraction with ethanol were analyzed by SPAGE (fig.1).

The crude residue was now extracted with 250 ml peroxide-free ether by standing for 3 × 24 h at 5°C. The supernatant ① + ② was separated from the residue ③ + ④ by pipetting; both were evaporated to dryness at 30°C i.vac. (scheme 1).

To the crude extract ① + ② and the crude residue ③ + ④ 30 ml and 150 ml $CHCl_3$/MeOH (2:1), respectively, were added, to remove residual lipids within 16 h. Four fractions were obtained which were analyzed by t.l.c. on Silicagel (Merck F 254) with $CHCl_3$/MeOH/H_2O/AcOH (50:30:4:8) [10]: ① a yellow oil with the main part of the lipids, ② a white precipitate, ③ a white extract

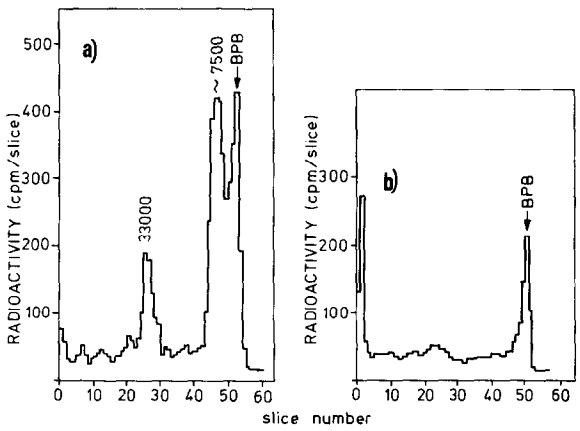


Fig.1. SDS polyacrylamide gel electrophoresis, at pH 5.0, without DTT of beef heart mitochondria, incubated with 6.9 nmol [^{14}C]NSPM/mg protein; (a) before and (b) after the extraction with ethanol. The gels were sliced without trichloroacetic acid treatment. Details are given in Materials and methods. BPB, bromphenol blue.

which contained most of the substances of ①, and ④ the crude, water-soluble residue which consisted mainly of saccharose + Tris–HCl and the [^{14}C]NSPM-binding protein.

The crude residue ④ was chromatographed on a column (2.5 × 100 cm) of Sephadex LH-20 with water as eluant. Four ultraviolet-absorbing fractions were obtained, of which only one was radioactive (fig.2) and migrated in t.l.c. on Silicagel described above (R_F 0.25).

2.6. *Measurement of radioactivity*

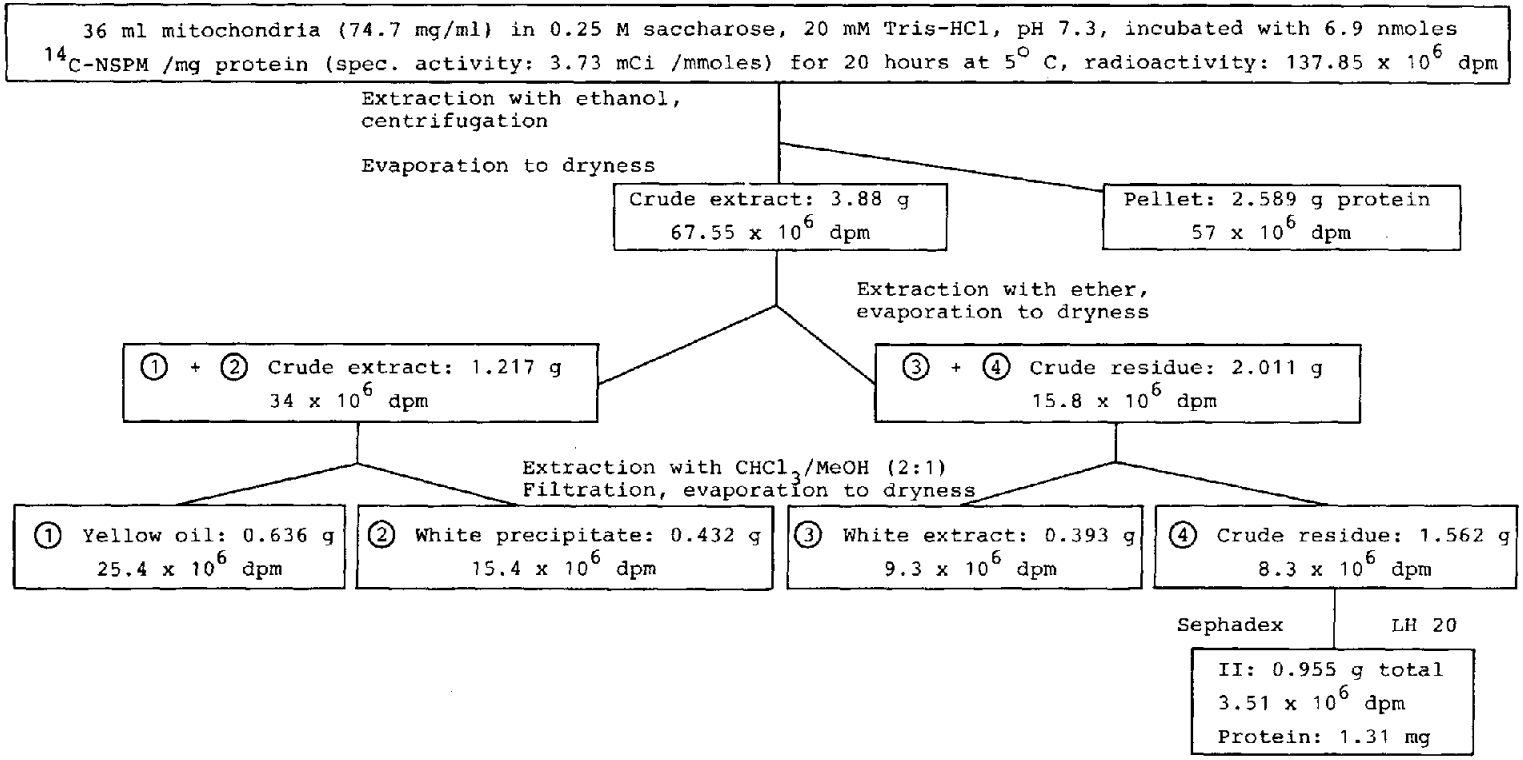
Radioactivity was measured in a Packard Tri-carb liquid-scintillation spectrometer; the mitochondrial membranes were solubilized with Protosol at 55°C within 3.5 h, the gels with 28% H_2O_2 at 60°C within 24 h [9]. [^{14}C]Toluene was used as internal standard.

3. Results

If beef heart mitochondria were incubated over night with 6.9 nmol [^{14}C]NSPM/mg protein (scheme 1), the minimal concentrations for complete inhibition of Site I coupling [1], three radioactive peaks could be detected in SPAGE analysis (fig.1a). After the extraction with ethanol (fig.1b) about 50% of

Scheme 1

The isolation of the ^{14}C-NSPM-binding protein from beef heart mitochondria



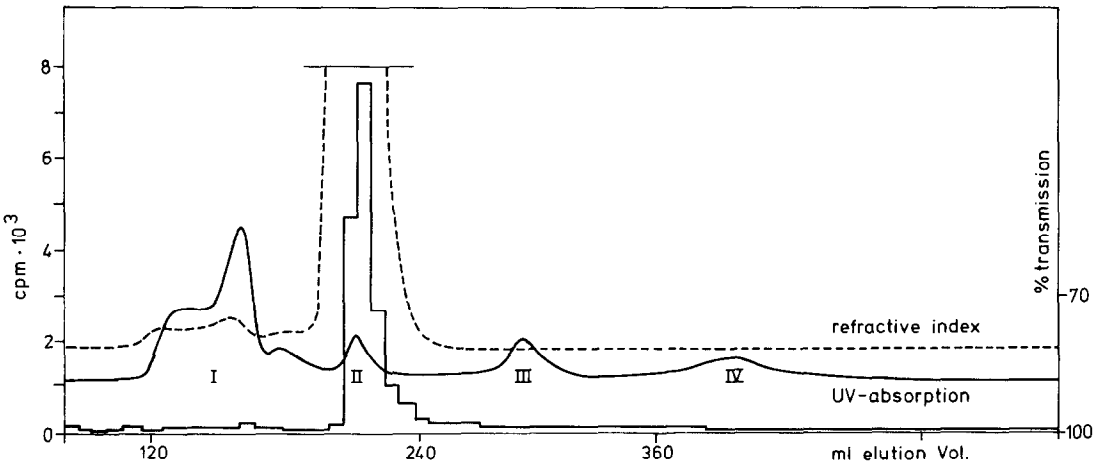


Fig.2. Column chromatography on Sephadex LH-20 of the crude residue ④ with water as eluant; details are given in Materials and methods. The dotted line represents the refractive indices measured by a diffraction refractometer; the main peak indicates the elution of saccharose.

the radioactivity was recovered in the supernatant (scheme 1). Evaporation to dryness, extraction with ether and a subsequent extraction of the obtained crude residue ③ + ④ (scheme 1) with $CHCl_3$/MeOH (2:1) yielded the crude residue ④, which was soluble in water. Only one radioactive fraction was obtained by column chromatography of ④ on Sephadex LH-20 with water as eluant (fig.2); in the same fraction saccharose was comigrated. Different radioactive fractions, prepared from mitochondria by the enzymatic method (subtilisin) as well by the mechanical method (high-speed blender), passed the column with the same elution volume and migrated the same distance in SPAGE analysis (fig.3). Yet the

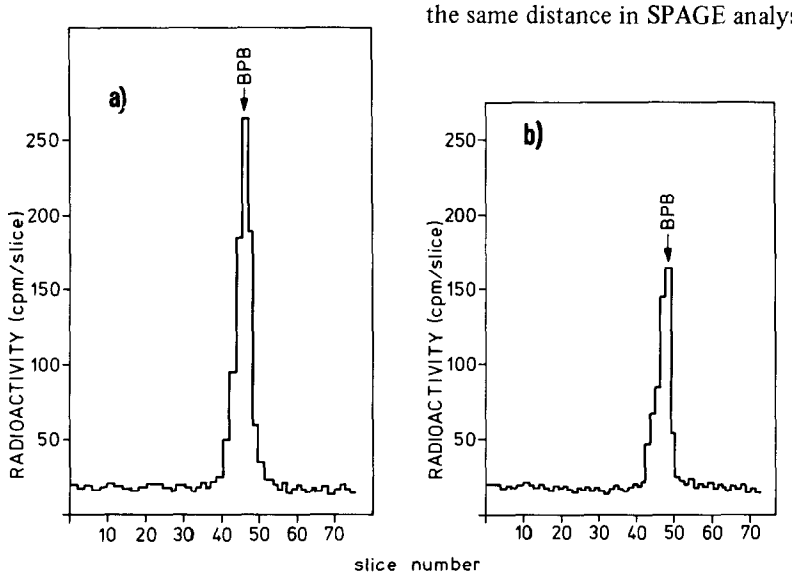


Fig.3. SDS–polyacrylamide gel electrophoresis, at pH 5.0, without DTT of the [^{14}C]NSPM-binding protein (Fraction II, fig.2), from mitochondria prepared: (a) by the high-speed blender method, (b) by the subtilisin method.

Table 1
Amino acid composition of the [^{14}C]NSPM protein isolated from different mitochondria preparations[a]

Amino acid	1	2
Asp	3.43	4.26
Thr	5.35	3.25
Ser	8.11	7.46
Glu	26.86	46.22
Pro	2.29	2.19
Gly	10.55	12.51
Ala	13.13	17.44
Val	6.39	1.52
Ile	4.05	0.56
Leu	7.27	1.12
Tyr	0.42	0.34
Phe	0.42	0.67
Lys	6.29	1.07
His	3.20	0.37
Arg	2.10	0.49

[a] The values reported are presented as mole percent

The protein was hydrolyzed in 6 N HCl for 24 h at 110°C. Methionine was destroyed during hydrolysis of these samples. The mitochondria were prepared: (1) by the subtilisin method; (2) by the high-speed blender method

two preparations differed in their amino acid composition to such an extent (table 1) that some proteolytic activity of residual subtilisin may be involved during the corresponding isolation procedure. Because of its unusual property, to be eluted in the staining procedure with Coomassie blue, the molecular weight could be estimated only by comparing different gels; it was lower than 13 000 (cytochrome *c*) and higher than 6000 (insulin).

4. Discussion

The inhibition of both, State 4 → State 3 transition as well as uncoupling by NSPM, was not caused by the inhibition of the glutamate-hydroxyl exchange [11], because the same effects were found with β-hydroxybutyrate. Inhibition of electron transport could be excluded, because NSPM as well as the corresponding succinimide showed this effect but with about 100 nmol/mg protein, which was ascribed to a second, pure lipophilic interaction of NSPM [1,12,13]. Therefore both, inhibition of Site I coupling as well as of the uncoupling were interpreted as the action of the isolated [^{14}C]NSPM-binding protein, though the second labelled protein (mol. wt ∿ 33 000) (fig.1a) could be the uncoupler-binding protein [13]. Yet the stimulation of State 4 respiration at Site III by NSPM could be uncoupled by dicoumarol [1]. Therefore it was reasonable to assume that the NSPM-binding protein alone was involved in the energy transfer between Complex I and the ATP synthase near to the electron transport chain.

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